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Behavior of *Yersinia enterocolitica* in the Presence of the Bacterivorous *Acanthamoeba castellanii*

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Free-living protozoa play an important role in the ecology and epidemiology of human-pathogenic bacteria. In the present study, the interaction between *Yersinia enterocolitica*, an important food-borne pathogen, and the free-living amoeba *Acanthamoeba castellanii* was studied. Several cocultivation assays were set up to assess the resistance of *Y. enterocolitica* to *A. castellanii* predation and the impact of environmental factors and bacterial strain-specific characteristics. Results showed that all *Y. enterocolitica* strains persist in association with *A. castellanii* for at least 14 days, and associations with *A. castellanii* enhanced survival of *Yersinia* under nutrient-rich conditions at 25°C and under nutrient-poor conditions at 37°C. Amoebae cultivated in the supernatant of one *Yersinia* strain showed temperature- and time-dependent permeabilization. Intraprotozoan survival of *Y. enterocolitica* depended on nutrient availability and temperature, with up to 2.8 log CFU/ml bacteria displaying intracellular survival at 7°C for at least 4 days in nutrient-rich medium. Transmission electron microscopy was performed to locate the *Yersinia* cells inside the amoebae. As *Yersinia* and *Acanthamoeba* share similar ecological niches, this interaction identifies a role of free-living protozoa in the ecology and epidemiology of *Y. enterocolitica*.

Yersinia enterocolitica is the third most frequently reported food-borne pathogen in the European Union, with 7,017 reported human cases in 2011 (1). Yersiniosis occurs mainly in infants and preschoolers and is characterized by acute enteritis with fever and diarrhea. In adolescents and adults, pseudoappendicitis, sequelae as arthritis and erythema nodosum, and septicemia can also occur (2, 3). Most infections are caused by *Y. enterocolitica* bioserotypes 4/O:3 and 2/O:9, which all harbor a virulence plasmid (pYV) that is required for full pathogenicity in humans (1, 4). This virulence plasmid is optimally expressed at 37°C but can become lost during laboratory cultivation (5, 6).

Most *Y. enterocolitica* infections in humans are associated with the handling and consumption of raw or undercooked pork products (1). Pigs are considered the primary reservoir of human-pathogenic *Y. enterocolitica*, as they harbor these pathogens in the tonsils and gastrointestinal lymphoid tissue (7, 8). However, many factors related to the epidemiology of *Y. enterocolitica*, such as environmental sources and transmission routes, remain indistinct (9).

Free-living protozoa are unicellular, heterotrophic, eukaryotic microorganisms ubiquitous in natural and anthropogenic aquatic and terrestrial environments, including food-related environments (10–13). Free-living protozoa, including members of the amoebal genus *Acanthamoeba*, graze on bacteria and are therefore considered important bacterial predators. However, various bacteria are able to evade protozoan ingestion and/or digestion and can even benefit from the association with protozoa (14). Some bacteria enhance their survival by extracellular association (15), although others are taken up and enter a membrane-bound vacuole, resisting protozoan digestion (16–18). Both associations result in survival, and even multiplication, of the bacteria in the presence of its predator. The intraprotozoan location further represents a shelter for bacteria against physical and chemical environmental conditions (16, 19). Moreover, free-living protozoa serve as a vehicle for the colonization of new habitats (16) and hosts (20). Free-living protozoa also play a role in the selection of bacterial virulence traits and adaptation to survival in macrophages (21–24). In addition, gene transfer between intraprotozoan bacteria has been reported (25).

Yersinia enterocolitica and free-living protozoa share the same ecological niches, such as water (26, 27), vegetables (28, 29), and anthropogenic environments, e.g., domestic refrigerators (13, 30). The role of free-living protozoa in the ecology and epidemiology of food-borne pathogens such as *Campylobacter*, *Salmonella*, and *Escherichia coli* has already been documented (31–33), but for *Y. enterocolitica*, only brief descriptions of intraprotozoan replication, probably followed by the killing and digestion of the bacterial strain (34), and increased resistance to free chlorine in their early intraprotozoan lifestyle (19) have been reported so far.

To further elucidate the ecology and epidemiology of this common pathogen, evaluation of the importance of its association with free-living protozoa, and the influence of environmental factors and *Yersinia* strain characteristics thereon, is needed.

The objectives of the present study were to assess the impact and the nature of the associations between *Y. enterocolitica* and free-living protozoa. Therefore, *in vitro* coculture experiments using the model protozoon *Acanthamoeba castellanii* and *Yersinia* strains with different bacterial characteristics were performed under different environmental conditions. These coculture experiments (i) evaluated the ability of *Y. enterocolitica* to survive or grow in association with the bacterivorous *Acanthamoeba* under different conditions, (ii) assessed if the yersiniae could survive or grow extracellularly on factors released by the amoebae and vice versa, and (iii) assessed if *Y. enterocolitica* cells were resistant to

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amoebal digestion, if they could survive or grow intracellularly, and exactly where they were located inside *A. castellanii*.

MATERIALS AND METHODS

Cultivation of *Acanthamoeba castellanii*. *Acanthamoeba castellanii* amoebae (ATCC 30234; American Type Culture Collection) were maintained axenically in proteose peptone yeast extract glucose (PYG) medium (ATCC recipe [http://www.lgcstandards-atcc.org/]) at 25°C in 75-cm^2 culture flasks. Light microscopic observations and plating of culture samples onto plate count agar (PCA; Bio-Rad, Hercules, CA, USA), which were incubated at 30°C for 48 h, were performed to verify the axenicity of the culture. For coculture experiments, stationary-growth-phase amoebae (3.5 days old) forming a monolayer were used. The adherent amoebae were harvested by tapping of the flasks and subsequent centrifugation of the solution (300 × *g* for 5 min), washed with Page's amoeba saline (PAS; ATCC recipe), and resuspended in PYG or PAS medium, depending on the experiment to be performed. For further interaction studies, the *Acanthamoeba* culture was diluted to ca. 5×10^5 living trophozoites/ml. The actual cell numbers were determined with a Füchs-Rosenthal chamber (Blaubrand, Wertheim, Germany). The membrane integrity and thus also the cell viability of the *Acanthamoeba* trophozoites were assessed by using the trypan blue exclusion assay (35).

Cultivation of *Yersinia enterocolitica* strains. Four *Y. enterocolitica* strains, isolated between 2010 and 2011, were used in this study: strain YeH3$^+$ (bioserotype 4/O:3; pYV$^+$) and its plasmid-cured derivate YeH3$^-$ were isolated from the stool of a 1-year-old boy, strain YeH9$^-$ (bioserotype 2/O:9; pYV$^-$) was isolated from the stool of a 43-year-old woman, and strain YeM3$^+$ (bioserotype 4/O:3; pYV$^+$) was isolated from minced pork. All strains were maintained in glycerol at −20°C. Strains were cultivated in tryptone soya broth (TSB; Bio-Rad, Hercules, CA, USA) at 37°C for 24 h to activate the pYV virulence plasmid (36). At the stationary growth phase, bacteria were harvested by centrifugation (10,000 × *g* for 5 min), washed in PAS, and resuspended in PYG or PAS (depending on the experimental setup). For use in further experiments, the bacterial suspension was diluted to ca. 5×10^7 CFU/ml by using data from *Y. enterocolitica* growth curves. The exact number of viable bacteria was determined after plating of a serial dilution of the suspension onto PCA and 48 h of incubation at 30°C. Before the start of the experiments and after each coculture experiment, the bioserotype and the presence of the virulence plasmid of each *Y. enterocolitica* strain were verified by biochemical testing and PCR analysis, as described previously by Van Damme et al. (37).

Coculture experiments. Coculture experiments with *A. castellanii* and the four *Y. enterocolitica* strains were performed in both nutrient-rich (PYG) and nutrient-poor (PAS) media (34, 35, 38) at 7°C, 25°C, and 37°C. These nutrient and temperature conditions were used to mimic natural conditions (e.g., aquatic areas [39]), food-related environmental conditions (e.g., refrigerators and temperatures and organic loads in food processing areas [13, 40]), and the mammalian body temperature.

Persistence of *Yersinia* in coculture with *Acanthamoeba castellanii*. The ability of *Y. enterocolitica* to survive in association with bacterivorous amoebae was assessed by persistence assays. With these assays, no distinction could be made regarding whether recovered bacteria were internalized in the amoebae or were adherent or free-living extracellular bacteria. *Acanthamoeba castellanii* cells were seeded into 12-well plates (2 ml/well at a concentration of ca. 5×10^5 living trophozoites/ml PYG or PAS), and plates were incubated at 25°C for 2 h to allow amoebal settlement and adhesion. The medium was then gently removed and replaced by 2 ml *Y. enterocolitica* suspension (ca. 5×10^5 or 5×10^7 CFU/ml, dissolved in PYG or PAS) to achieve a multiplicity of infection (MOI) of ca. 1 bacterium per amoeba and ca. 100 bacteria per amoeba, respectively. Plates were centrifuged at 50 × *g* for 5 min to enhance cell contact and incubated at 7°C, 25°C, or 37°C. Monocultures of *A. castellanii* and of each *Yersinia* strain were included as controls. At days 1, 2, 3, 6, 7, 10, and 14, the cells were harvested by scraping the wells. Amoebal cell integrity was visually evaluated by light microscopy and the trypan blue exclusion assay. The number of viable *Yersinia* cells was determined after plating of serial dilutions of the cell suspension onto PCA and incubation for 48 h at 30°C.

Effect of released factors on microbial survival and growth. The ability of *Y. enterocolitica* to survive and/or grow in association with *A. castellanii* without direct interaction with the amoebae, i.e., extracellularly on factors released by the amoebae and vice versa, was determined by supernatant assays. Therefore, *A. castellanii* (ca. 5×10^5 cells/ml) and *Yersinia* strains (ca. 5×10^7 CFU/ml) were harvested after 5 min of centrifugation at 300 × *g* and 5 min at 10,000 × *g*, respectively. The supernatants of both the bacterial and amoebal monocultures were filter sterilized (0.22-μm Nalgene Syringe cellulose acetate membrane; Thermo Scientific, Langenselbold, Germany), and the pH of the bacterial supernatant was measured (WTW pH 330i; WTW, Weilheim, Germany). Subsequently, the *Yersinia* cells were resuspended in the supernatant of the amoeba cultures and vice versa. One milliliter of each *Yersinia* or *A. castellanii* suspension was inoculated into 12-well plates. As controls, amoebae suspended in TSB medium (growth medium of the *Yersinia* cultures) and *Y. enterocolitica* strains suspended in PYG medium (growth medium of *Acanthamoeba*) were included. All plates were incubated at 7°C, 25°C, and 37°C. After 3 h and 1, 2, and 3 days of incubation, cells were harvested by scraping the wells. The number of viable *Yersinia* cells and the amoebal concentration and cell integrity were determined as described above.

Intraprotozoan survival of *Y. enterocolitica*. The ability of *Y. enterocolitica* to survive inside *A. castellanii*, and the number of intracellular *Yersinia* cells, was assessed by gentamicin protection assays. Cocultures and amoebal monocultures were set up as described above for the persistence assays. After 2 h of (co)cultivation, the medium was gently removed, and the cells were washed with PAS to minimize extracellular bacteria. Afterwards, 4 ml of gentamicin sulfate solution (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 100 μg/ml PYG (34) was added to each well, and the plates were incubated for 1 h at 7°C, 25°C, or 37°C to kill extracellular *Y. enterocolitica* cells. In previous tests, this gentamicin treatment was effective in killing 99.97 to 100% of the *Y. enterocolitica* cells, with no effect on the viability of *Acanthamoeba castellanii* (data not shown). For the experiments with PAS, gentamicin suspended in PYG was also used for initial killing of the extracellular bacteria, as this treatment was most effective (34). After incubation, wells were washed with PAS, and 4 ml gentamicin maintenance solution (20 μg gentamicin/ml PAS or PYG, depending on the experimental setup) was added to each well. This moment was defined as the 0-h time point (i.e., 3 h after initial setup of the [co]culture). The plates were further incubated at 7°C, 25°C, or 37°C, and after 1, 2, 3, and 4 days, the number of amoebae and the intra- and extra-amoebal bacterial counts were determined. For the extra-amoebal bacterial counts, the supernatant was removed and plated onto PCA plates. Afterwards, wells were washed with PAS, 4 ml PYG or PAS was added to each well, and the amoebae were harvested by cell scraping. This cell suspension was subsampled for further analysis: 0.5 ml was used for enumeration and viability testing of amoebae as described above, 2 ml of the cell suspension was used for transmission electron microscopy (TEM) analysis (see below), and the remaining amoebal suspension (1.5 ml) was used to determine the intra-amoebal bacterial counts. For the latter, the amoebae were lysed with 0.5% sodium deoxycholate for 5 min at room temperature, which has been shown to result in effective lysis of the amoebae without affecting intracellular bacteria (data not shown). The suspension was plated onto PCA plates. All plates were incubated at 30°C for 48 h, after which the extra- and intracellular *Yersinia* cells were counted.

Localization of intraprotozoan *Y. enterocolitica*. TEM was applied to determine the exact subcellular location of intraprotozoan *Y. enterocolitica*. The amoebae from the coculture and control wells from the gentamicin protection assays (second aliquot) were harvested by centrifugation at 300 × *g* for 5 min and serially fixed with glutaraldehyde in sodium cacodylate buffer (pH 7.4) as follows: 0.8%, 1.25%, and, finally, 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (each concentration included a 20-min incubation at 4°C under continuous rotation [13 rpm] [Dynal MX-1; Life Technologies, Belgium]). Cells were then rinsed three

times for 10 min in 0.05 M sodium cacodylate buffer. Postfixation took place in 1% osmium tetroxide in the same buffer for 1 h at room temperature. Afterwards, the cells were rinsed twice with distilled water and were stepwise dehydrated, using ethanol series of increasing concentrations (15 to 100% ethanol, for 10 min each). The cells were subsequently impregnated with a low-viscosity embedding medium (Spurr's resin [41]), and polymerization was performed at 70°C for 8 h. Samples were sectioned on a Reichert Ultracut S instrument (Leica, Vienna, Austria), first semithin (0.5 μm) until the region of interest was reached, after which ultrathin (70-nm) sections were made. Semithin sections were studied by using a Wild light microscope (Heerbrugg, Switzerland). Ultrathin sections were studied with a JEOL JEM-1010 transmission electron microscope (JEOL Ltd., Tokyo, Japan) operating at 60 kV, and pictures were digitized by using a Ditabis system (Pforzheim, Germany).

Statistical analysis. All assays (persistence, supernatant, and gentamicin protection) were performed in triplicate, except for the persistence assay at an MOI of 1:1. Statistical data analysis was performed on the quantitative data by using Stata 11.0 software (Stata Corporation, College Station, TX, USA). A probability (P) value of <0.05 was required for statistical significance. Bacterial counts obtained by the persistence assays were $\log_{10}$ transformed and analyzed for each time point by using generalized least-squares regressions, with strains as random effects. Coculture and monoculture counts were compared for every combination of temperature and nutrient condition. To determine strain and/or temperature differences in the absolute number of living amoebae/bacteria (supernatant test) and of intraprotozoan *Yersinia* cells (gentamicin protection assays), negative binomial regressions were used, including strains as random effects where necessary.

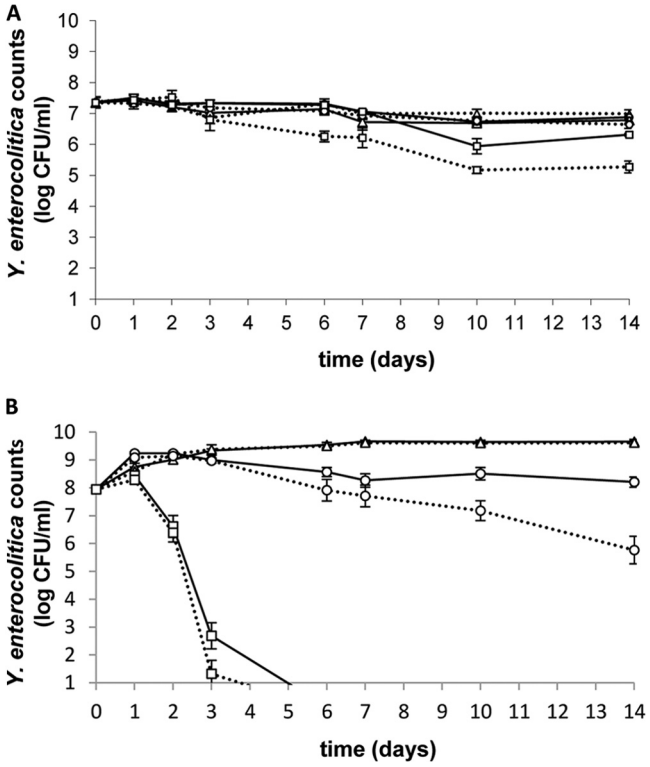

FIG 1 Persistence of *Y. enterocolitica* in coculture with *A. castellanii*. *Yersinia enterocolitica* cells were cultivated with (full line) or without (dotted line) *A. castellanii* in nutrient-poor PAS (A) or nutrient-rich PYG medium (B) at different temperatures (△, 7°C; ○, 25°C; □, 37°C) at an MOI of 100:1. Values represent the overall means of the four *Y. enterocolitica* strains of the three replicate experiments ± standard errors of the means.

RESULTS

Persistence of *Yersinia* in coculture with *Acanthamoeba castellanii*. In general, for each strain under each experimental condition (temperature and medium combination), the survival of *Y. enterocolitica* cells in both cocultures and controls was shown not to be strain dependent.

In the cocultures, under nutrient-poor conditions (PAS) (Fig. 1A), the number of viable yersiniae did not differ significantly between the different temperatures ($P > 0.05$). The number of viable *Yersinia* cells under both coculture and monoculture conditions at 7°C and 25°C remained almost constant during the 14-day monitoring period, with no significant differences between cocultures and monocultures ($P > 0.05$). At 37°C, the number of viable *Yersinia* cells in the monocultures decreased earlier than the ones in the cocultures, and by day 14, the number of viable *Yersinia* cells in the monoculture was significant smaller than that in the cocultures ($P < 0.001$).

Under nutrient-rich conditions (PYG) (Fig. 1B), the number of viable yersiniae was significantly higher in cocultures at 7°C and 25°C than in cocultures at 37°C after 1 day and significantly higher at 7°C than at 25°C after 6 days ($P < 0.001$). However, similar differences between temperatures were obtained in the *Yersinia* monocultures. For each temperature, an initial bacterial growth of 0.5 to 1.5 log CFU/ml was observed for both cocultures and controls (Fig. 1B). From day 3 onwards, a *status quo* of viable *Yersinia* cells was observed in both co- and monocultures at 7°C (ca. 9.5 log CFU/ml) and in the cocultures at 25°C (ca. 8.5 log CFU/ml), whereas bacterial viability in the monocultures at 25°C decreased. At 37°C, no viable *Yersinia* cells could be recovered after day 3 in both the co- and monocultures. At 7°C and 37°C, no significant difference in viable *Yersinia* counts was detected between the cocultures and the monocultures, whereas at 25°C, the number of viable *Yersinia* cells in cocultures was significantly higher than that in the monocultures from day 6 onwards ($P < 0.05$).

Under each condition (i.e., specific strain, temperature, and medium combinations), light microscopy revealed no visual difference in amoebal cell integrity and density between cocultures and amoebal monocultures during the 14-day monitoring period. Under most conditions, the amoebae stayed adherent, and pseudopodia and vacuoles were visible, in both co- and monocultures. In contrast, in PAS medium at 7°C and 25°C, a gradual transformation of trophozoites into resting cysts was observed after 3 and 6 days, respectively, under both coculture and monoculture conditions. However, after 14 days, living trophozoites were still detected. At 37°C, amoebal viability declined, and cyst formation and the presence of amoebal cell debris were observed from days 3 to 4 onwards under both coculture and amoeba monoculture conditions. Furthermore, from day 6 onwards, no trophozoites were detected at 37°C.

To increase the amoebal grazing pressure on *Yersinia*, persistence assays at an MOI of 1:1 were also performed at 7°C, 25°C, and 37°C under nutrient-poor conditions, which resulted in observations similar to those reported above for an MOI of 100:1 (data not shown).

Effect of released factors on microbial survival and growth. In general, the survival and growth of yersiniae inoculated in amoebal supernatant were similar to those seen in the controls. For both, an initial growth of ca. 1.0 to 1.5 log CFU/ml was ob-

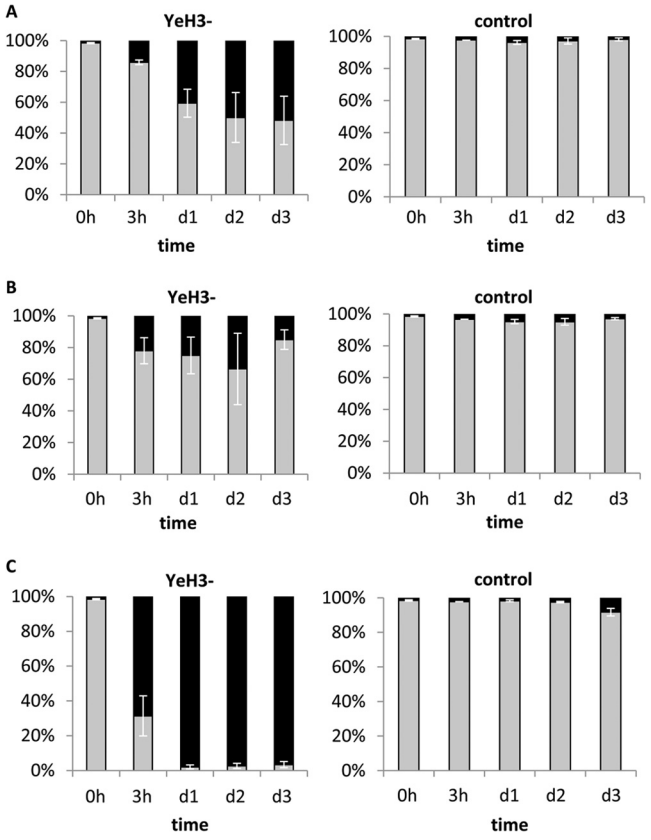

FIG 2 Effect of supernatant from *Y. enterocolitica* strain YeH3$^-$ on *A. castellanii*. Shown are percentages of living (indicated in gray) and permeabilized (in black) amoebae related to the total amoebal count when cultivated in the cell-free supernatant of a *Y. enterocolitica* culture (strain YeH3$^-$) and incubated at 7°C (A), 25°C (B), and 37°C (C). Amoebae were cultivated in the growth medium of the *Yersinia* culture (TSB) as a control. Bars represent the means of three replicate experiments ± standard errors of the means.

served at each temperature. At 7°C and 25°C, a slight increase of ca. 1 log CFU/ml was detected after time, while at 37°C, a rapid decrease in *Yersinia* viability of ca. 6 to 8 log CFU/ml was observed after 1 day (data not shown).

No differences in amoebal survival and growth between those amoebae cultivated in bacterial supernatant and those in control medium were observed, except for amoebae inoculated in the supernatant of *Yersinia* strain YeH3$^-$ (Fig. 2). Here, significantly lower absolute numbers of viable amoebae were detected in the bacterial supernatant than in the control after 3 h of incubation at 37°C ($P < 0.001$), after 2 days at 25°C ($P < 0.05$), and after 3 days at 7°C ($P < 0.001$). From 3 h onwards, the number of living amoebae under supernatant treatment conditions at 37°C was significantly lower than that under treatment conditions at 25°C and 7°C ($P < 0.01$). The total number of amoebae, i.e., the sum of permeabilized and viable amoebae, remained the same over time (ca. 5×10^5 cells/ml).

The initial pH of TSB medium before inoculation was 7.2 ± 0.1, but it decreased to 5.2 when YeH3$^-$ was cultivated in TSB. The pH-lowering effect of the other strains was less pronounced (pH 5.6 to 6.5). Acidified TSB medium alone (HCl; pH 5.2) also caused permeabilization at 7°C and 37°C but to a much lesser extent (i.e., maximum of 28% permeabilized cells after day 3) than for the amoebae cultivated in the YeH3$^-$ supernatant (data not shown).

Intraprotozoan survival of *Y. enterocolitica*. Under each experimental condition, amoebal counts and cell integrity remained constant over time in both cocultures and amoeba monocultures. In nutrient-poor medium (PAS) at 25°C and 37°C, no viable intracellular *Yersinia* bacteria could be recovered after 1 day. At 7°C, no extracellular *Yersinia* bacteria were recovered at the 0-h time point, after treatment with 100 μg/ml gentamicin. However, recovery of extracellular *Yersinia* bacteria after 1 day, probably due to the failure of the gentamicin maintenance solution (20 μg/ml) to kill extracellular bacteria in PAS at a low temperature, compromised the exact determination of the amount of intracellular *Yersinia*.

In nutrient-rich medium (PYG), *Y. enterocolitica* could survive inside *A. castellanii* (Fig. 3). Over time, all *Y. enterocolitica* strains survived intracellularly for at least 4 days at 7°C, with 0.71 to 2.8 log CFU/ml detected inside the amoebae. At 25°C and 37°C, the number of intracellular *Yersinia* bacteria decreased, with low or no bacterial recovery at the end of the experiment. At each time point, the number of intracellular *Y. enterocolitica* bacteria in coculture with *Acanthamoeba* was significantly higher at 7°C than at 25°C ($P < 0.001$) and 37°C ($P \leq 0.001$).

Although the initial inoculation concentration was the same under each experimental condition (combination of strain and temperature), counts were already significantly different between temperature treatments at time point zero (i.e., after a 2-h feeding period followed by a 1-h gentamicin treatment). Strains YeH3$^+$ and YeM3$^+$ had similar concentrations at 0 h, whereas the number of viable yersiniae of strain YeH3$^-$ was almost 1 log CFU/ml lower. Replicate counts at the 0-h time point of strain YeH9$^-$ at 7°C varied from 0.53 to 4.50 log CFU/ml (although with similar inoculation levels at the start of the experiment), whereas replicates of all other strain-temperature combinations varied much less.

Localization of intraprotozoan *Y. enterocolitica*. At each time point, transmission electron microscopy was performed to determine the intra-amoebal location of the bacteria. Pseudopodia and food vacuoles were visible, indicating that the experimental coculture conditions favored normal amoebic activity. After 3 days of cocultivation, the intraprotozoan bacteria were located in the cytoplasm of the amoeba but were not visibly surrounded by an amoebal vacuole membrane (Fig. 4). The host endoplasmic reticulum (ER) was located close to the intracellular bacterium.

DISCUSSION

The present study showed that *Acanthamoeba castellanii* enhances *Yersinia enterocolitica* survival under certain environmental conditions. As *Yersinia* and *Acanthamoeba* share similar ecological and anthropogenic niches, this interaction identifies a potential role of free-living protozoa in the ecology and epidemiology of *Y. enterocolitica*. Although *Acanthamoeba* is a bacterivorous free-living species that actively grazes on bacteria, with an estimated ingestion rate of up to 700 bacteria per amoeba per hour (42), in the persistence assays, no decreased bacterial viability was observed in the cocultures with *A. castellanii* during the 14-day monitoring period. This proves that *Y. enterocolitica* could survive in the presence of *A. castellanii*, although no distinction could be made regarding whether the recovered bacteria were internalized in the amoebae (i.e., resistant to amoebal digestion) or were adherent or free-living extracellular bacteria (i.e., resistant to amoebal uptake).

Moreover, the persistence assays showed that interaction with *A. castellanii* enhances the survival of *Y. enterocolitica* under

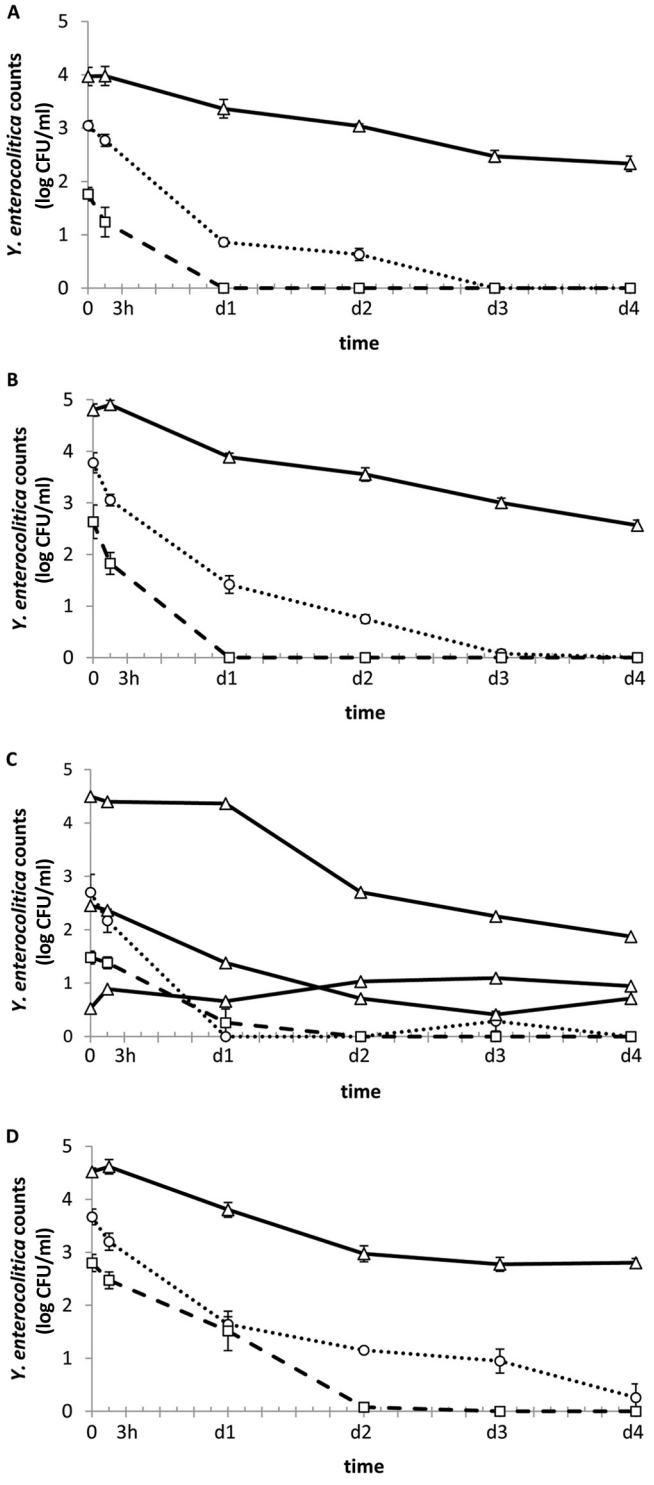

FIG 3 Intracellular viable counts of *Y. enterocolitica* at different incubation temperatures. *Acanthamoeba castellanii* and *Y. enterocolitica* strains YeH3$^-$ (A), YeH3$^+$ (B), YeH9$^-$ (C), and YeM3$^+$ (D) were cocultivated in nutrient-rich medium and incubated at different temperatures (△, 7°C; ○, 25°C; □, 37°C). Values represent the means of three replicate experiments ± standard errors of the means.

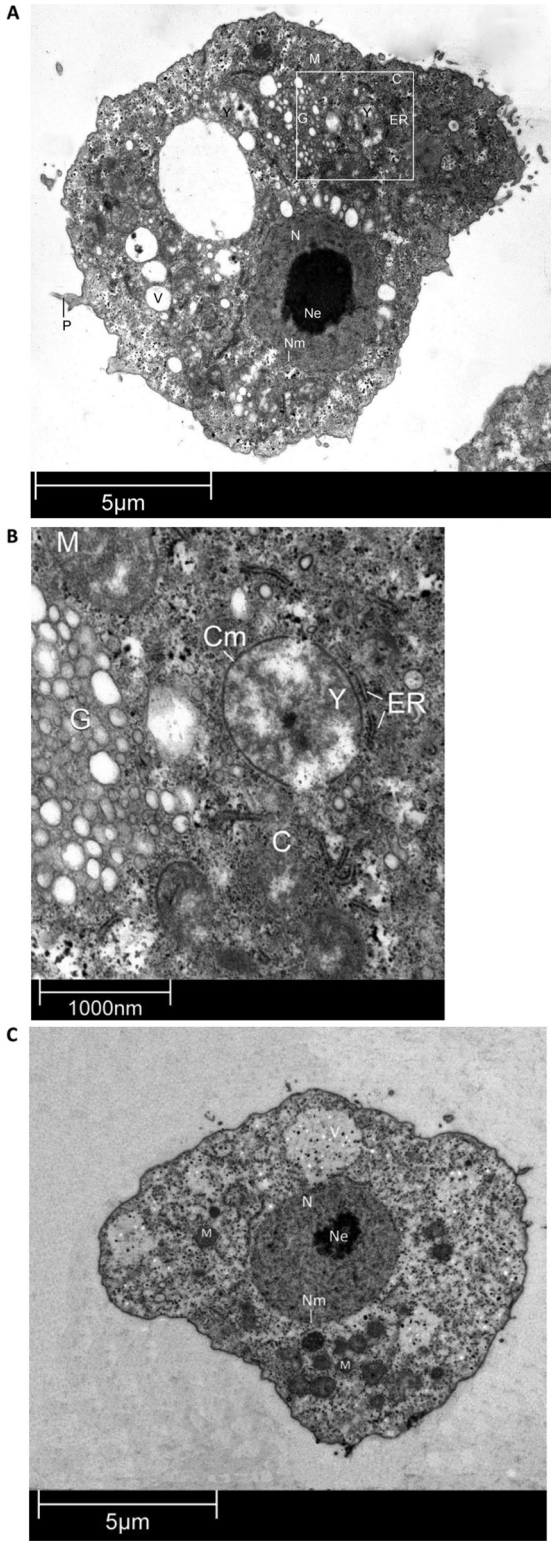

FIG 4 *Acanthamoeba castellanii* trophozoite with internalized *Yersinia enterocolitica*. Shown are TEM micrographs of *A. castellanii* incubated for 3 days with *Y. enterocolitica* strain YeH3$^-$ in nutrient-rich medium at 7°C (A and B) and of an *A. castellanii* monoculture control (C). P, pseudopodium; V, food vacuole; C, cytoplasm; Nm, nucleus membrane; N, nucleus; Ne, nucleolus; M, mitochondrion; Y, *Yersinia enterocolitica*; ER, endoplasmic reticulum; G, globules; Cm, bacterial cell membrane.

nutrient-rich conditions at 25°C and under nutrient-poor conditions at 37°C. However, the presence of cell debris in both cocultures and amoebal monocultures at the latter temperature indicates amoebal lysis, presumably due to nutrient depletion and a

supraoptimal temperature. A similar decrease in amoebal viability under co- and monoculture conditions was also reported by Baré et al. and Greub et al. (35, 43). The formed amoebal cell debris may in turn be used by *Yersinia* as a nutrient source, explaining the better survival in the cocultures at 37°C than in the monocultures. The fact that no *Yersinia* cells could be detected in both cocultures and monocultures after 3 days of incubation in nutrient-rich medium at 37°C is presumably due to the high metabolic activity of *Yersinia* under these conditions, which led to rapid nutrient depletion.

The present study also revealed that low temperatures and high nutrient availability favor intraprotozoan survival. These conditions are met in industrial food processing settings, at home as well as in natural environments. Although *Yersinia* is a psychrotrophic bacterium and can withstand low temperatures without amoebal protection (bacterial monoculture controls in persistence assays) (44), the association can enhance *Y. enterocolitica* survival by physically protecting against chlorine treatment. Chlorine is a compound commonly used to disinfect water and food processing equipment, and increased resistance of intraprotozoan *Y. enterocolitica* against chlorine has been reported (19).

In general, larger numbers of intracellular bacteria were recovered at lower temperatures and for a longer period of time. This is in parallel with a recently reported study where a *Yersinia* strain was not recovered from *Acanthamoeba polyphaga* after 2 days at 30°C (34). The high intraprotozoan survival rate observed at 7°C could be due to slower amoebal phagocytic digestion (45); bacterial circumvention of amoebal digestion by escaping into the amoebal cytosol, as observed by TEM; or both. Further research is needed to elucidate *Yersinia*'s intracellular survival mechanisms.

The number of viable intracellular *Yersinia* bacteria decreased with time. This could be due to (partial) bacterial digestion, bacterial release in the environment, and subsequent bacterial killing by gentamicin or to a switch to a viable-but-noncultivable (VBNC) state of the intracellular *Yersinia* bacteria (46). Partial intraprotozoan digestion was described previously for other bacterial pathogens (35, 47–49). Linking back toward the results of the persistence assay, whereby no reduction in the number of viable bacteria was observed under most cocultivation conditions, another explanation could be that intraprotozoan *Yersinia* cells are released or trigger their release in the extra-amoebal environment (34). In the gentamicin protection assay, these extracellular bacteria are killed by the gentamicin maintenance concentration, whereas extracellular growth/survival is possible for yersiniae in the persistence assay. In the gentamicin protection assay, no amoebal growth was observed, which confirmed that the amoebae did not appear to obtain adequate nutrients from *Yersinia* to increase their concentration.

Strain variation in the intracellular survival capacity was detected from the start of the experiments onwards, which could be due to variation in the ability of the strains to invade or to become internalized by *Acanthamoeba* or to their intrinsic intracellular survival capacities. This study indicates that the presence of the *Yersinia* virulence plasmid favors bacterial uptake and/or intracellular survival, as at time point zero, the number of intracellular yersiniae was higher for the plasmid carrying the YeH3$^+$ strain than for its plasmid-cured derivative (YeH3$^-$).

For the first time, TEM was used to visualize the exact subcellular location of *Yersinia* inside *A. castellanii*. Images show that the intracellular yersiniae were located in the cytosol and were not visibly surrounded by an amoebal vacuole membrane. This may indicate that *Y. enterocolitica* is able to circumvent the normal digestion pathway by escaping from the food vacuole to the cytosol. This postingestional adaptation mechanism to avoid protozoan digestion was also described previously for members of the genus *Rickettsia* (18). In addition, the *Y. enterocolitica* cells were located close to the host endoplasmic reticulum (ER), which may be advantageous, as the bacteria have easy access to newly synthesized host proteins. Further research is necessary to reveal if the colocalization of host ER and intracellular yersiniae is coincidental or if there exists a *Yersinia* mechanism to repose the host ER, as described previously for *Legionella pneumophila* (50, 51).

In the supernatant assays, the supernatant of strain YeH3$^-$ (4/O:3; pYV$^-$) had a temperature-dependent permeabilizing effect on the amoebae. It is not clear if this reflects a direct or indirect effect on amoebal cell membrane integrity. During bacterial cultivation at the start of the experiments, the pH of this supernatant had already decreased compared to the pH of the supernatants of the other strains. However, the permeabilizing effect could not be explained by a pH reduction as such. As this permeabilizing effect was not observed in the persistence assays, a hypothesis could be that direct cell contact between amoebae and bacteria may activate a host defense mechanism to prevent permeabilization. Alternatively, the effect can be concentration dependent, as washing steps in the persistence assay protocol could have lowered the number of permeabilizing factors. Further research is needed to identify the cause of this permeabilization (influenced by temperature) and the observed pH drop, with attention to the production of a potential bacterial toxin or a metabolite with antiprotozoan activity (14).

The association of *Y. enterocolitica* with protozoa has relevant ecological and epidemiological implications. Besides enhancing *Y. enterocolitica* survival in their presence, being intra- or extracellular, free-living protozoa have the potential to act as a reservoir, vector, infection route, biological gym, and evolutionary crib for intracellular *Yersinia enterocolitica*, as previously described for other pathogenic bacteria (17, 24, 52, 53).

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REFERENCES

1. **EFSA.** 2013. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011. EFSA J. **11:**3129–3379.
2. **Bottone EJ.** 1999. *Yersinia enterocolitica*: overview and epidemiologic correlates. Microbes Infect. **1:**323–333.
3. **Verhaegen J, Charlier J, Lemmens P, Delmee M, Van Noyen R, Verbist L, Wauters G.** 1998. Surveillance of human *Yersinia enterocolitica* infections in Belgium: 1967-1996. Clin. Infect. Dis. **27:**59–64.
4. **Bottone EJ.** 1997. *Yersinia enterocolitica*: the charisma continues. Clin. Microbiol. Rev. **10:**257–276.
5. **Riley G, Toma S.** 1989. Detection of pathogenic *Yersinia enterocolitica* by using Congo red-magnesium oxalate agar medium. J. Clin. Microbiol. **27:**213–214.
6. **Farmer JJ, III, Carter GP, Miller VL, Falkow S, Wachsmuth IK.** 1992. Pyrazinamidase, CR-MOX agar, salicin fermentation-esculin hydrolysis, and D-xylose fermentation for identifying pathogenic serotypes of *Yersinia enterocolitica*. J. Clin. Microbiol. **30:**2589–2594.
7. **Van Damme I, Habib I, De Zutter L.** 2010. *Yersinia enterocolitica* in slaughter pig tonsils: enumeration and detection by enrichment versus direct plating culture. Food Microbiol. **27:**158–161.
8. **Nesbakken T, Eckner K, Hoidal HK, Rotterud OJ.** 2003. Occurrence of

Yersinia enterocolitica and *Campylobacter* spp. in slaughter pigs and consequences for meat inspection, slaughtering, and dressing procedures. Int. J. Food Microbiol. **80:**231–240.
9. **Fredriksson-Ahomaa M, Stolle A, Korkeala H.** 2006. Molecular epidemiology of *Yersinia enterocolitica* infections. FEMS Immunol. Med. Microbiol. **47:**315–329.
10. **Baré J, Sabbe K, Van Wichelen J, van Gremberghe I, D'Hondt S, Houf K.** 2009. Diversity and habitat specificity of free-living protozoa in commercial poultry houses. Appl. Environ. Microbiol. **75:**1417–1426.
11. **Baré J, Houf K, Verstraete T, Vaerewijck M, Sabbe K.** 2011. Persistence of free-living protozoan communities across rearing cycles in commercial poultry houses. Appl. Environ. Microbiol. **77:**1763–1769.
12. **Vaerewijck MJ, Sabbe K, Baré J, Houf K.** 2008. Microscopic and molecular studies of the diversity of free-living protozoa in meat-cutting plants. Appl. Environ. Microbiol. **74:**5741–5749.
13. **Vaerewijck MJ, Sabbe K, Van Hende J, Baré J, Houf K.** 2010. Sampling strategy, occurrence and diversity of free-living protozoa in domestic refrigerators. J. Appl. Microbiol. **109:**1566–1578.
14. **Matz C, Kjelleberg S.** 2005. Off the hook: how bacteria survive protozoan grazing. Trends Microbiol. **13:**302–307.
15. **Akya A, Pointon A, Thomas C.** 2009. Viability of *Listeria monocytogenes* in co-culture with *Acanthamoeba* spp. FEMS Microbiol. Ecol. **70:**20–29.
16. **Berk SG, Faulkner G, Garduno E, Joy MC, Ortiz-Jimenez MA, Garduno RA.** 2008. Packaging of live *Legionella pneumophila* into pellets expelled by *Tetrahymena* spp. does not require bacterial replication and depends on a Dot/Icm-mediated survival mechanism. Appl. Environ. Microbiol. **74:**2187–2199.
17. **Greub G, Raoult D.** 2004. Microorganisms resistant to free-living amoebae. Clin. Microbiol. Rev. **17:**413–433.
18. **Whitworth T, Popov VL, Yu XJ, Walker DH, Bouyer DH.** 2005. Expression of the *Rickettsia prowazekii* pld or tlyC gene in *Salmonella enterica* serovar Typhimurium mediates phagosomal escape. Infect. Immun. **73:**6668–6673.
19. **King CH, Shotts EB, Jr, Wooley RE, Porter KG.** 1988. Survival of coliforms and bacterial pathogens within protozoa during chlorination. Appl. Environ. Microbiol. **54:**3023–3033.
20. **Snelling WJ, Stern NJ, Lowery CJ, Moore JE, Gibbons E, Baker C, Dooley JS.** 2008. Colonization of broilers by *Campylobacter jejuni* internalized within *Acanthamoeba castellanii*. Arch. Microbiol. **189:**175–179.
21. **Bruggemann H, Cazalet C, Buchrieser C.** 2006. Adaptation of *Legionella pneumophila* to the host environment: role of protein secretion, effectors and eukaryotic-like proteins. Curr. Opin. Microbiol. **9:**86–94.
22. **Koubar M, Rodier MH, Garduno RA, Frere J.** 2011. Passage through *Tetrahymena tropicalis* enhances the resistance to stress and the infectivity of *Legionella pneumophila*. FEMS Microbiol. Lett. **325:**10–15.
23. **Cirillo JD, Falkow S, Tompkins LS.** 1994. Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. Infect. Immun. **62:**3254–3261.
24. **Cirillo JD, Falkow S, Tompkins LS, Bermudez LE.** 1997. Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. Infect. Immun. **65:**3759–3767.
25. **McCuddin ZP, Carlson SA, Rasmussen MA, Franklin SK.** 2006. *Klebsiella* to *Salmonella* gene transfer within rumen protozoa: implications for antibiotic resistance and rumen defaunation. Vet. Microbiol. **114:**275–284.
26. **Falcão JP, Brocchi M, Proenca-Modena JL, Acrani GO, Correa EF, Falcão DP.** 2004. Virulence characteristics and epidemiology of *Yersinia enterocolitica* and yersiniae other than *Y. pseudotuberculosis* and *Y. pestis* isolated from water and sewage. J. Appl. Microbiol. **96:**1230–1236.
27. **Hausmann K, Hülsmann N, Radek R.** 2003. Protistology. Schweizerbart'sche verlagbuchhandlung Stuttgart, Stuttgart, Germany.
28. **Cocolin L, Comi G.** 2005. Use of a culture-independent molecular method to study the ecology of *Yersinia* spp. in food. Int. J. Food Microbiol. **105:**71–82.
29. **Vaerewijck MJ, Sabbe K, Baré J, Houf K.** 2011. Occurrence and diversity of free-living protozoa on butterhead lettuce. Int. J. Food Microbiol. **147:**105–111.
30. **Jackson V, Blair I, McDowella D, Kennedy J, Bolton D.** 2007. The incidence of significant foodborne pathogens in domestic refrigerators. Food Control **18:**346–351.
31. **Axelsson-Olsson D, Svensson L, Olofsson J, Salomon P, Waldenstrom J, Ellstrom P, Olsen B.** 2010. Increase in acid tolerance of *Campylobacter jejuni* through coincubation with amoebae. Appl. Environ. Microbiol. **76:**4194–4200.
32. **Douesnard-Malo F, Daigle F.** 2011. Increased persistence of *Salmonella enterica* serovar Typhi in the presence of *Acanthamoeba castellanii*. Appl. Environ. Microbiol. **77:**7640–7646.
33. **Alsam S, Jeong SR, Sissons J, Dudley R, Kim KS, Khan NA.** 2006. *Escherichia coli* interactions with *Acanthamoeba*: a symbiosis with environmental and clinical implications. J. Med. Microbiol. **55:**689–694.
34. **Anacarso I, de Niederhausern S, Messi P, Guerrieri E, Iseppi R, Sabia C, Bondi M.** 2012. *Acanthamoeba polyphaga*, a potential environmental vector for the transmission of food-borne and opportunistic pathogens. J. Basic Microbiol. **52:**261–268.
35. **Baré J, Sabbe K, Huws S, Vercauteren D, Braeckmans K, van Gremberghe I, Favoreel H, Houf K.** 2010. Influence of temperature, oxygen and bacterial strain identity on the association of *Campylobacter jejuni* with *Acanthamoeba castellanii*. FEMS Microbiol. Ecol. **74:**371–381.
36. **Rohde JR, Luan XS, Rohde H, Fox JM, Minnich SA.** 1999. The *Yersinia enterocolitica* pYV virulence plasmid contains multiple intrinsic DNA bends which melt at 37°C. J. Bacteriol. **181:**4198–4204.
37. **Van Damme I, Berkvens D, Baré J, De Zutter L.** 2013. Influence of isolation methods on the occurrence of plasmid-carrying *Yersinia enterocolitica* serotype O:3 in slaughter pig tonsils, faeces and carcass surface swabs. Int. J. Food Microbiol. **164:**32–35.
38. **Axelsson-Olsson D, Olofsson J, Svensson L, Griekspoor P, Waldenstrom J, Ellstrom P, Olsen B.** 2010. Amoebae and algae can prolong the survival of *Campylobacter* species in co-culture. Exp. Parasitol. **126:**59–64.
39. **Berry D, Horn M, Xi C, Raskin L.** 2010. *Mycobacterium avium* infections of *Acanthamoeba* strains: host strain variability, grazing-acquired infections, and altered dynamics of inactivation with monochloramine. Appl. Environ. Microbiol. **76:**6685–6688.
40. **Vaerewijck MJ, Sabbe K, Baré J, Spengler HP, Favoreel HW, Houf K.** 2012. Assessment of the efficacy of benzalkonium chloride and sodium hypochlorite against *Acanthamoeba polyphaga* and *Tetrahymena* spp. J. Food Prot. **75:**541–546.
41. **Spurr A.** 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26:**31–43.
42. **Corsaro D, Müller K-D, Michel R.** 2013. Molecular characterization and ultrastructure of a new amoeba endoparasite belonging to the Stenotrophomonas maltophilia complex. Exp. Parasitol. **133:**383–390.
43. **Greub G, La Scola B, Raoult D.** 2003. *Parachlamydia acanthamoeba* is endosymbiotic or lytic for *Acanthamoeba polyphaga* depending on the incubation temperature. Ann. N. Y. Acad. Sci. **990:**628–634.
44. **Goverde RL, Huis in't Veld JH, Kusters JG, Mooi FR.** 1998. The psychrotrophic bacterium *Yersinia enterocolitica* requires expression of pnp, the gene for polynucleotide phosphorylase, for growth at low temperature (5 degrees C). Mol. Microbiol. **28:**555–569.
45. **Khan N.** 2009. *Acanthamoeba*: biology and pathogenesis. Caister Academic Press, Norfolk, England.
46. **Smith JJ, Howington JP, McFeters GA.** 1994. Survival, physiological response and recovery of enteric bacteria exposed to a polar marine environment. Appl. Environ. Microbiol. **60:**2977–2984.
47. **Pickup ZL, Pickup R, Parry JD.** 2007. Effects of bacterial prey species and their concentration on growth of the amoebae *Acanthamoeba castellanii* and *Hartmannella vermiformis*. Appl. Environ. Microbiol. **73:**2631–2634.
48. **Gaze WH, Burroughs N, Gallagher MP, Wellington EM.** 2003. Interactions between *Salmonella typhimurium* and *Acanthamoeba polyphaga*, and observation of a new mode of intracellular growth within contractile vacuoles. Microb. Ecol. **46:**358–369.
49. **Tezcan-Merdol D, Ljungstrom M, Winiecka-Krusnell J, Linder E, Engstrand L, Rhen M.** 2004. Uptake and replication of *Salmonella enterica* in *Acanthamoeba rhysodes*. Appl. Environ. Microbiol. **70:**3706–3714.
50. **Robinson CG, Roy CR.** 2006. Attachment and fusion of endoplasmic reticulum with vacuoles containing *Legionella pneumophila*. Cell. Microbiol. **8:**793–805.
51. **de Felipe KS, Glover RT, Charpentier X, Anderson OR, Reyes M, Pericone CD, Shuman HA.** 2008. *Legionella* eukaryotic-like type IV substrates interfere with organelle trafficking. PLoS Pathog. **4:**e1000117. doi: 10.1371/journal.ppat.1000117.
52. **Siddiqui R, Khan NA.** 2012. War of the microbial worlds: who is the beneficiary in *Acanthamoeba*-bacterial interactions? Exp. Parasitol. **130:**311–313.
53. **Thomas V, McDonnell G, Denyer SP, Maillard JY.** 2010. Free-living amoebae and their intracellular pathogenic microorganisms: risks for water quality. FEMS Microbiol. Rev. **34:**231–259.